*THE PROCESS OF INFECTION WITH BACTERIOPHAGE φX174, XXX. REPLICATION OF DOUBLE-STRANDED φX DNA**

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Abstract.—Intermediates involved in the replication of double-stranded φX174 RF DNA have been identified and partially characterized. Analysis of pulse-labeled RF DNA suggests that the synthesis of progeny RF molecules involves, in part, the addition of nucleotides to linear complementary strands on a circular parental strand as template, so as to produce intermediate DNA strands of greater than viral length. Electron microscopy reveals DNA rings with "tails" and "double rings," which could be the intermediate structures. A model is postulated for the replication process.

Semiconservative replication of the replicative form (RF)[1] of bacteriophage φX174 DNA is only observed for those RF molecules which are associated with an active "replication site."[2, 3] There are one to four replication sites per host cell,[2] dependent on the metabolic state of the cell. In cells infected with multiplicities of more than four phage per cell, only the parental RF molecules can participate in the semiconservative production of daughter RF molecules.[3] The replicating parental RF molecules are found in the membrane fractions of a cell extract while the replication products, the daughter RF molecules, are not bound to the membrane and are most probably free in the cytoplasm of the cell.[4]

These processes are observed during the eclipse period of a normal infection cycle and during abortive infection in the presence of 30–40 μg/ml chloramphenicol (when only daughter RF molecules, but no progeny single-stranded DNA molecules, are formed). In this paper we describe some experiments which give, in a preliminary way, more insight into the process of the semiconservative reproduction of double-stranded φX DNA molecules.

Experimental Procedures.—Bacteria and phages: The host strain *E. coli* H502 (hcr⁻ EndoI⁻thy⁻su⁻) was kindly provided by Dr. Hoffman-Berling. The lysis-defective mutant, φX*am*3, has been described.[5] The DNA of the extended host range, temperature-sensitive mutant of φX*ts*γh, was used as a biological marker. The preparation of P^{32}-φX*am*3 has been described.[6] The spheroplast assay was that of Guthrie and Sinsheimer.[7] Mitomycin pretreatment of the host cells to preferentially suppress the synthesis of host cell DNA has been described.[8]

Media: Minimal medium has been described by Knippers, Razin, Davis, and Sinsheimer.[9] Tris-EDTA is 0.05 *M* Tris-HCl–0.005 *M* EDTA, pH 8.

Infection procedure: E. coli H502 cells at a concentration of 5×10^8 cells/ml were incubated at 37° in the presence of mitomycin. After 30–40 min the cells were resuspended in fresh medium containing 1 μg/ml of thymine and 35 μg/ml of chloramphenicol. After 5 min at 37°, P^{32}-φX*am*3 in a multiplicity of 5–6 was added. Five minutes later the culture was placed in a 20° waterbath. After 20–25 min, H^3-thymidine (11 C/mmole, Schwarz Bioresearch) in a concentration of 0.03 mC/ml was added for various lengths of time as indicated in the *Results* section. Replication was terminated by pouring the culture into a container with an acetone-dry ice mixture at −40°. The cells were then

pelleted in the cold and washed two times with cold Tris-EDTA containing 0.05 *M* sodium azide.

Other techniques: The use of sarkosyl and pronase for the extraction of ϕX RF has been described.[9] Radioactively labeled DNA was precipitated with an equal volume of cold 10% trichloroacetic acid, collected on glass filter discs, dried, and counted in a scintillation spectrophotometer with a toluene base scintillator. All centrifuge techniques were those of Knippers and Sinsheimer.[10] Details will be given in the legends to the appropriate figures. Chromatography on columns of benzoylated, naphthoylated DEAE-cellulose was performed as previously described.[9, 13]

Results.—Pulse experiments: A total of 6×10^{10} H502 cells in 100 ml medium (1 μg/ml of thymine, 35 μg/ml of chloramphenicol) was infected with P^{32}-*am*3 (multiplicity 5). After 20 minutes at 20°, 2 mC H^3-thymidine were added for about 5 seconds. The extracted DNA was first sedimented through a neutral sucrose gradient (Fig. 1). Parental P^{32}-label appeared in three peaks, corresponding to single-stranded DNA (25*S;* originating, probably, from adsorbed phage particles which had not penetrated), to RFI (21*S*), and to RFII (16.5*S*). The H^3-pulse label cosedimented predominantly with the P^{32}-RFII, while a faster-sedimenting shoulder of H^3-labeled DNA sedimented with 20–22*S*. Less than 10 per cent of the total acid-precipitable H^3-radioactivity sedimented with 10*S*, perhaps analogous to Okazaki's fragments[11] of replicating *E. coli* DNA or T4 bacteriophage DNA.

Fractions 8–16 of the gradient of Figure 1, containing most of the pulse label, were combined and dialyzed against Tris-EDTA to remove the sucrose. The DNA in these fractions was then concentrated by isopropanol precipitation in the presence of denatured calf thymus DNA (0.2 mg) as carrier. The recovered DNA (about 70%) was denatured (by addition of 0.02 vol 10 *N* NaOH) and sedimented through an alkaline sucrose gradient (Fig. 2). About 65 per cent of the P^{32}-parental label sedimented with the rate of the infectious DNA marker

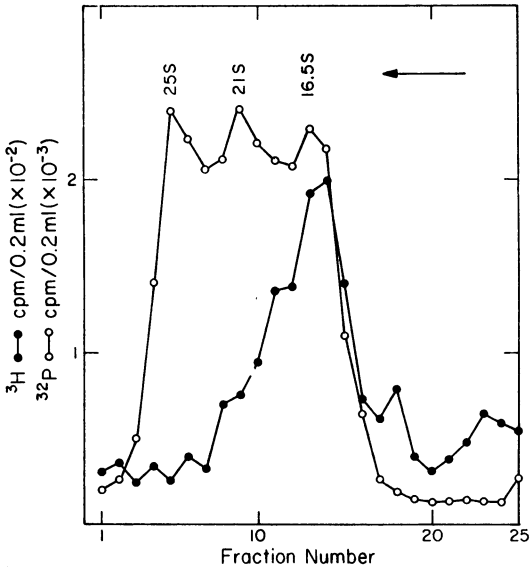

FIG. 1.—Sedimentation analysis of pulse-labeled, double-stranded ϕX DNA at neutral pH. Sedimentation was performed through linear 20–5% sucrose gradients in 0.3 *M* NaCl–Tris-EDTA, in the SW25.1 rotor of the Spinco centrifuge, for 14 hr at 25,000 rpm at 4°. The arrow indicates the direction of sedimentation. Fraction volume: 1.2 ml.

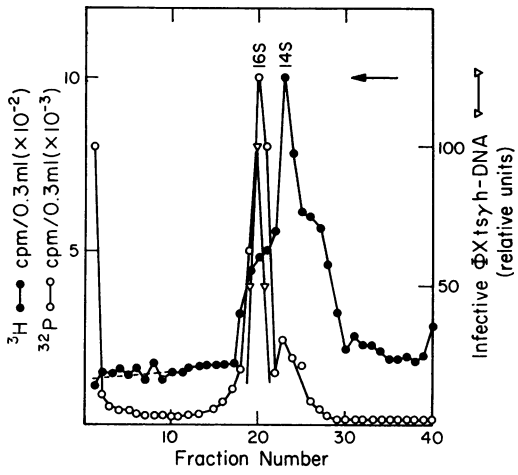

FIG. 2.—Sedimentation of pulse-labeled ϕX RF through an alkaline sucrose gradient. Alkali-denatured DNA in a 0.5-ml volume was sedimented through a 28-ml gradient (20–5% sucrose in 0.3 *M* NaOH–0.01 *M* EDTA) in the SW25.1 rotor for 27 hr at 25,000 rpm at 5°. The arrow indicates the direction of sedimentation. Fraction volume: 0.7 ml.

(16*S*) and was presumably circular DNA (*vide infra*); approximately 15 per cent of the P^{32}-counts were on the bottom of the centrifuge tube (probably in fast-sedimenting, denatured RFI); the remaining P^{32}-radioactivity (15%) sedimented with 14*S*, the sedimentation coefficient of linear ϕX DNA molecules of unit length.

The H^3-pulse label was distributed in a more complex manner. The most prominent peak of H^3-labeled DNA sedimented with 14*S*, the rate of linear ϕX DNA strands of unit length. Faster-sedimenting pieces of pulse-labeled DNA appeared in a leading shoulder of this main peak, with sedimentation rate up to 18*S*, the rate expected for double-length ϕX DNA strands. DNA pieces smaller than unit length were also found, sedimenting with *S* values below 14*S*. In the experiment of Figure 2 as well as in other similar experiments, more pulse label appeared in the fractions of "smaller" DNA pieces than in the "larger" (35% vs. 15% of the total H^3-acid-precipitable DNA).

All H^3-labeled DNA species in the gradient of Figure 2 were degraded by exonuclease I.[12] The only enzyme-resistant DNA found was the P^{32}-labeled circular 16*S*-parental strand. Repeatedly, some 5–20 per cent of the parental P^{32}-DNA has been found to be in the linear form (14*S*). Whether such molecules are artifacts of extraction or whether they represent intermediate states in the ϕX replication cycle cannot yet be decided. The variability observed in the relative amount of linear parental strands in different experiments would favor the first possibility.

To obtain more information about the nature of the various pulse-labeled DNA strands, appropriate samples were recovered from the alkaline sucrose gradient and analyzed by sedimentation to equilibrium in a CsCl gradient at pH 12.5. The 14*S* material and the pulse-labeled material in the leading shoulder ($S > 14$) usually gave satisfactory distribution profiles at equilibrium; however, no clear results could be obtained by equilibrium centrifugation of the shorter ($S < 14$) fragments. As can be seen from Figure 3, the H^3-14*S* peak contained material forming bands at the densities of complementary and viral DNA strands in

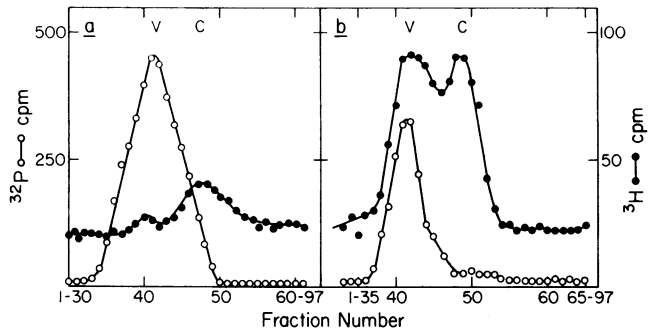

FIG. 3.—Equilibrium centrifugation of DNA in CsCl at pH 12.5. A 2.7-ml volume (initial density: 1.75 gm/ml) was centrifuged for 24 hr at 38,000 rpm and for 48 hr at 30,000 rpm in the SW65 rotor of the Spinco centrifuge at 14°. 97 fractions were collected directly on GF/A glass fiber filters, dried, and counted. (*a*) Centrifugation of fractions 18–20 of Fig. 2 (*S* values >14*S*); (*b*) Centrifugation of fraction 22–24 of Fig. 2 (*S* values around 14*S*). Density increases from right to left; *V*, viral strand; *C*, complementary strand.

approximately equal amounts, whereas the larger DNA strands ($S > 14$) were found predominantly at the density corresponding to complementary strand material.

Chromatography on benzoylated, naphthoylated DEAE-cellulose (BNC): BNC columns have been used successfully to separate replicating DNA complexes from resting molecules.[9, 13] Replicating DNA complexes appear to contain single-stranded regions[14, 15] and are more firmly bound to the column. They can be eluted only with caffeine, whereas the nonreplicating, wholly double-stranded DNA molecules are eluted with NaCl. The data of Table 1 indicate that this distinction was also true for the replicating ϕX RF: The proportion of the total incorporated acid-precipitable radioactivity eluted only with caffeine decreased with increasing length of H^3-pulse, suggesting that the immediately replicating ϕX RF complexes were present in a partially denatured form.

Figure 4 shows the elution pattern of ϕX RF, extracted from a P^{32}-*am*3-

TABLE 1. *Chromatography of pulse-labeled ϕX RF on benzoylated, naphthoylated DEAE-cellulose.*

Pulse time (sec)	Recovered P^{32} (cpm)	Per cent in NaCl eluate	Per cent in caffeine eluate
10	5,210	32	68
40	5,050	34	66
120	4,870	29	71
	Recovered H^3 (cpm)		
10	1,180	28	72
40	3,705	53	47
120	14,765	69	31

H502 cells (5×10^8/ml) in 30 ml minimal medium (35 μg/ml of chloramphenicol) were infected with P^{32}-*am*3 in a multiplicity of 6. After 20 min at 30°, H^3-thymidine was added and equal samples were withdrawn from the culture at the indicated times. The extracted ϕX DNA was chromatographed as described in the legend to Fig. 4. Final recovery of acid-precipitable radioactivity was 83, 79, and 86% after the 10-, 40-, and 120-sec pulses, respectively.

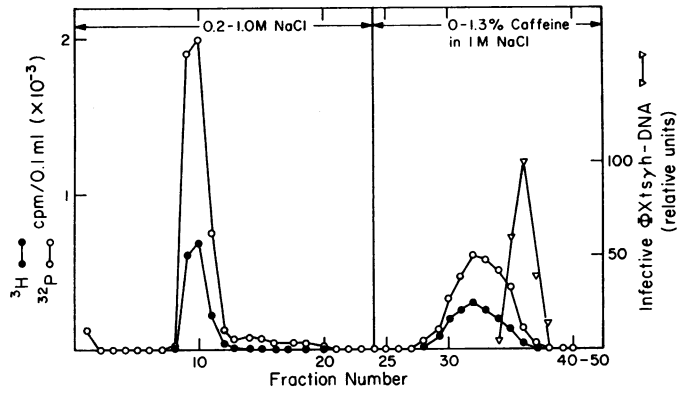

FIG. 4.—Chromatography on a column of benzoylated, naphthoylated DEAE-cellulose. Column dimensions: 2 cm × 3 cm. The column was washed with 50 ml 0.2 *M* NaCl–Tris-EDTA before the DNA sample was added to the column. Elution: (*i*) linear gradient of 0.2 *M* NaCl to 1 *M* NaCl in Tris-EDTA; (*ii*) linear gradient of 0 to 1.3% caffeine in 1 *M* NaCl–Tris-EDTA. Flow rate, 0.5 ml/min; fraction volume, 2 ml; recovery of input acid-precipitable radioactivity, 87%.

infected cell culture which had been pulse-labeled with H^3-thymidine for 40 seconds at 30°. About 50 per cent of the P^{32}-parental DNA and H^3-pulse-labeled DNA eluted in the NaCl gradient, probably representing nonreplicating parental RF molecules. The other half of the labeled DNA was eluted with caffeine, in fractions well separated from the single-stranded marker ϕX DNA. Our interpretation that the NaCl-eluted DNA represents nonreplicating DNA and that the caffeine-eluted material contains replicating RF complexes is supported by the alkaline sucrose gradients shown in Figures 5 and 6.

Centrifugal analysis of the salt-eluted DNA (Fig. 5) showed that most of the P^{32}-parental label was present in fast-sedimenting denatured RFI (34*S*) molecules. Part of the H^3-labeled DNA also sedimented with 34*S;* however, the greater portion (about 60%) was denatured to circular (16*S*) and linear (14*S*) compo-

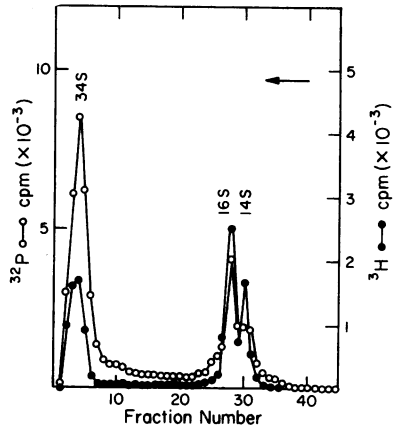

FIG. 5.—Sedimentation analysis of salt-eluted, denatured DNA. The salt-eluted DNA of the chromatogram of Fig. 4 was concentrated by CsCl equilibrium centrifugation. After dialysis to remove the CsCl, a 0.3-ml sample was denatured (0.03 ml 10 *N* NaOH) and sedimented through a linear sucrose gradient (20% to 5% sucrose in 0.3 *M* NaOH–0.01 *M* EDTA, pH 12.5) in the SW41 rotor of the Spinco centrifuge for 6 hr at 40,000 rpm at 5°. The arrow indicates the direction of sedimentation.

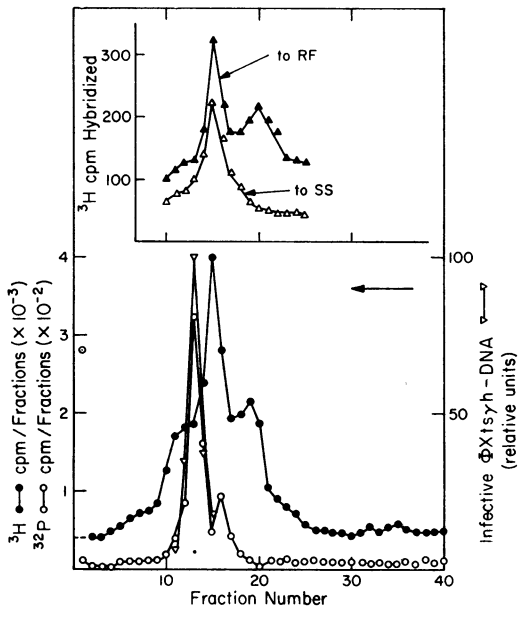

FIG. 6.—Sedimentation analysis of the denatured, caffeine-eluted DNA. The caffeine-eluted DNA of the chromatogram of Fig. 4 was concentrated by a neutral CsCl equilibrium centrifugation, dialyzed, and then denatured with alkali. The denatured DNA was sedimented as described in Fig. 5, except that the centrifugation time was 9 hr. One half of each fraction was precipitated on a GF/A glass fiber filter and counted. Portions of the other half were neutralized and used for the DNA-DNA hybridization procedure of Waarnar and Cohen,[17] employing Millipore filters loaded either with denatured RF (4 μg/filter) or with phage-extracted viral DNA (*SS*, 4 μg/ml). The total H^3-radioactivity retained on these filters after three cycles of washings with a low ionic strength buffer is shown in the inset.

nents from RFII molecules. These early replication products have been described previously[16] and are composed of equal amounts of circular viral and complementary strands and of linear viral and complementary strands, respectively.

The distribution of label observed after alkaline sedimentation of the caffeine-eluted DNA (Fig. 6) was similar to that shown for the pulse-labeled DNA of Figure 2. More than 90 per cent of the parental P^{32}-labeled DNA strands was circular (16*S*). The H^3-pulse label appeared in a prominent 14*S*-peak and in faster-sedimenting DNA pieces ($S > 14$) with a shoulder at 18*S* (where φX DNA of twice the unit length is expected to sediment). A shallow peak of H^3-labeled material sedimenting slower than unit length linear DNA ($S < 14$) was also present.

The nature of the various pulse-labeled DNA strands was studied by a DNA-DNA annealing technique.[17] The results (inset, Fig. 6) indicated that most of the slower-sedimenting pieces were composed of sequences present only in the viral strand of the RF.

Electron microscopy:[18] The caffeine-eluted DNA was also examined by electron microscopy.[19] The results of a more systematic study of replicating RF intermediates will be published elsewhere. Two morphological states of replicating RF have been found in the caffeine eluate, as shown in Figure 7: rings with tails and "double rings." Tails longer than one unit length of φX DNA were not detected. From these preliminary data it is not possible to decide which of the two forms represent the normal morphology of RF intermediates. Hirt's work on replicating polyoma DNA molecules[20] would suggest that the "double ring" structure is the usual form of replicating "small" DNA rings.

Discussion and Summary.—Despite several uncertainties, it appears that a list of the components of replicating RF molecules should include (1) circular parental

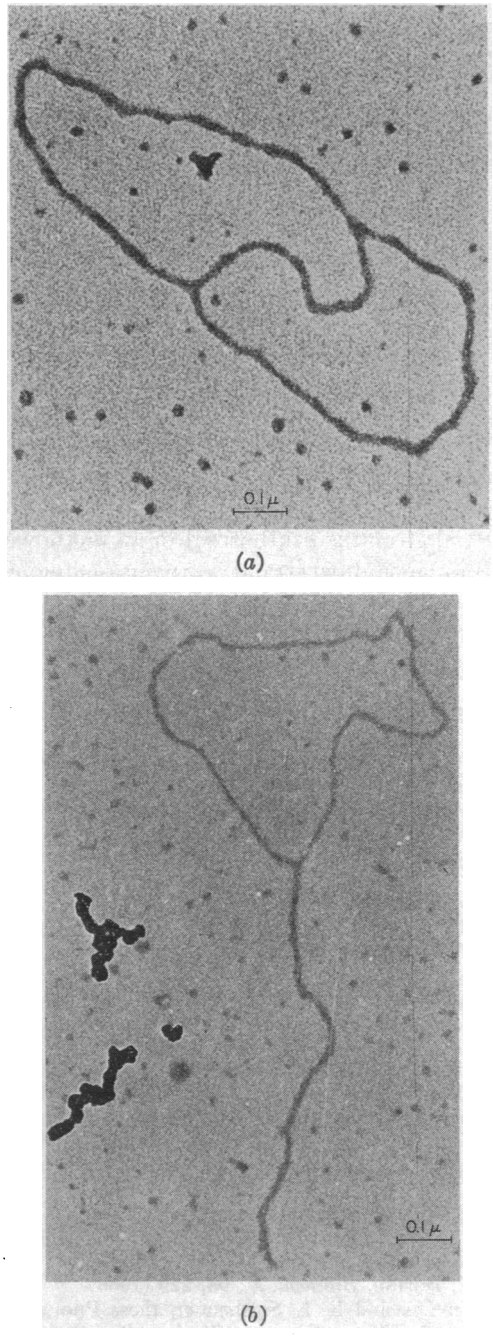

FIG. 7.—Electron micrograms of replicating ϕX RF molecules.

DNA strands; (2) linear complementary strands which probably grow by addition of nucleotides to one of their ends; and (3) DNA pieces of newly synthesized viral DNA strands which are shorter than one unit length ϕX DNA.

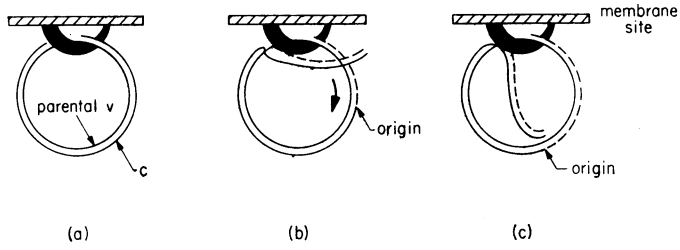

FIG. 8.—Model of double-stranded ϕX RF replication. (*a*) At beginning of replication; (*b*) partially replicated: nascent "tail" free; (*c*) partially replicated: nascent "tail" bonded to origin; *v*, viral strand; *c*, complementary strand.

If the enzymatic replication apparatus is located on the host cell membrane,[21] it can be assumed that the circular parental strand rotates through that complex (Fig. 8). Nucleotides are added to the 3'-OH-terminus of the complementary strand, thereby producing complementary strand pieces longer than one unit length. The new viral strand is synthesized in an unknown way on the emerging old part of the complementary strand. An entangling of the nascent double-stranded DNA tail should be avoided by a loose association (via a protein?) of the end with the "origin" of the DNA circle, as suggested by the electron micrograms.

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[1] RF is the intracellular double-stranded "replicative form" DNA of ϕX174. RFI is the supercoiled DNA duplex; RFII is the "nicked" relaxed form. The RF, containing the DNA of the infecting virus, is called parental RF. DNA strands, homologous to those found in the viral particle, are called viral strands; their complementary partners are called complementary strands.

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[18] The assistance generously provided by R. Davis and D. Clayton, California Institute of Technology, is gratefully acknowledged. The electron micrograms shown here are those of R. Davis.

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